

METHOD TO DETECT ANTIBODIES SPECIFIC FOR TYPE-2 TURKEY ASTROVIRUS

Background of the Invention

5 Acute gastroenteritis is one of the world's most significant disease problems. An estimated 3 to 5 million people die each year from gastroenteritis, mostly in the developing world (Glass et al., 2001). In the United States, viral gastroenteritis is one of the most common acute illnesses, second only to viral respiratory diseases (Glass et al., 2001). Although several viruses cause gastroenteritis, the most
10 clinically relevant include rotaviruses, caliciviruses, astroviruses, and enteric adenoviruses (Cukor et al., 1984).

 Viral gastroenteritis occurs in both an endemic and epidemic fashion, based on the routes of transmission and host response. The most common endemic viruses are group A rotaviruses, enteric adenoviruses, astroviruses and the Sapporo-like
15 viruses (caliciviruses) (Glass et al., 2001). These infections are virtually universal in the first years of life. It is believed that during early childhood, immunity develops to these agents providing protection against recurring infection and explaining the decrease in cases in older children and adults (Kurtz et al., 1978; Kurtz et al., 1979; Mitchell, 2002). Epidemic viruses are best characterized by the
20 Norwalk-like viruses (calicivirus) and the group B rotaviruses. These viruses affect people of all ages, and outbreaks are typically linked to contaminated water and/or food (Goodgame, 2001).

 Astroviruses, small round, non-enveloped viruses, typically 28-30 nm in diameter, are implicated in epidemics, usually associated with an institutional
25 setting like a hospital, retirement community, or military base, and they have been isolated from shellfish linked to food borne disease (Matsui et al., 2001).

 Astroviruses have been reported to cause acute disease in the young of multiple species, including humans, cattle, sheep, cats, dogs, deer, chickens, turkeys, and ducks (Bridger, 1980; Gough et al., 1984; Harboav et al., 1987; Madeley et al.,
30 1975; McNulty et al., 1988; Snodgrass et al., 1977; Tzipon et al., 1981; Williams, 1980; Woode et al., 1978), and multiple serotypes have been described for human, bovine, and turkey astroviruses.

Astroviruses are commonly recognized as a problem in turkeys. Turkey astrovirus (TAsV) was first described by McNulty et al. (1980) in poult in the United Kingdom suffering from diarrhea and increased mortality. In the United States, TAsV was first identified in the 1980s (TAsV-1), and shown to be widely distributed (Reynolds et al., 1986; Reynolds et al., 1987b; Saif et al., 1985). Reynolds et al. (1987b) demonstrated that astroviruses could be isolated from 78% of diseased turkey flocks, more than any other virus identified. TAsV is generally associated with self-limiting mild enteritis, transient growth depression, moderate increases in mortality (Jonassen et al., 2003; Koci et al., 2000; McNulty et al., 1980; Reynolds, 1991; Reynolds et al., 1987b; Yu et al., 2000) and malabsorption (Reynolds et al., 1986; Reynolds et al., 1987a; Thouvenelle et al., 1995a; Thouvenelle et al., 1995b).

Recently, a TAsV isolate, TAsV-2, that is associated with poult enteritis and mortality syndrome (PEMS), was characterized (Koci et al., 2000). PEMS is a multifactorial, highly infectious emerging disease that affects young turkeys, typically between 7 and 28 days of age. The disease was first described in 1991 in an area along the western North Carolina/South Carolina border (Barnes et al., 1997). A PEMS-like disease has been described in most turkey producing states across the United States (Barnes et al., 1997; Brandenberger, 1999), and has been estimated to cost the turkey industry over \$100 million (Brandenberger, 1999). TAsV-2 is genetically and immunologically distinct from previously described isolates (Koci et al., 2000).

Strict containment is the only known method of preventing and controlling infections with any of the known astroviruses. Infected flocks, especially those that exhibit severe loss in viability and production, need to be treated with the utmost concern for biosecurity, strictly adhering to the principles discussed in Zander & Mallinson (1991). Astroviruses are extremely stable in the environment and resistant to inactivation by most routinely used disinfectants (Kurtz et al., 1980; Abad et al., 1997; Schultz-Cherry et al., 2001) similar to chicken anemia virus or foot-and-mouth disease virus. For instance, partially purified TAsV-2 remains infectious following treatment with a panel of commercial disinfectants, including

10% bleach. TAstV-2 is also very heat stable, resisting inactivation following treatment at 60°C for 10 minutes, and resistant to low pH (Schultz-Cherry et al., 2001). These findings suggest that, once a poultry production facility has been infected with astrovirus, complete sanitation of all materials and restricted access to facilities by personnel is required to contain the outbreak to an affected farm.

The combination of age susceptibility and highly stable virions suggests that multiple age farms may help prolong the period of poor production as older birds may recover and no longer exhibit clinical signs but still harbor virus. For example, new poults routinely develop enteritis soon after being placed in “cleaned” houses on farms with multiple aged birds (Edens & Doerfler, 1999). The most practical prevention method is to use strict biosecurity prophylactically. A nominal investment of time and energy spent on keeping each farm pathogen-free could greatly reduce the likelihood of contracting an astrovirus infection, and likewise periods of prolonged poor production.

Until recently the most common method to identify astrovirus infection in birds was electronmicroscopy (EM) (Reynolds, 1991). However, only 10% of particles may exhibit the 5- or 6-pointed starlike morphology making it difficult to accurately identify astroviruses using direct EM, especially when there are very few viral particles present (Caul & Appleton, 1982; Reynolds, 1991; Matsui & Greenberg, 2001). Because of this limitation, Reynolds (1991) suggested using immune EM (IEM) to encourage viral aggregation, however, the addition of purified antibody or convalescent sera to a virus sample can actually mask characteristic physical features or fail to detect new serotypes (Matsui & Greenberg, 2001).

Currently, the one diagnostic tool available for TAstV-2 is a reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Koci et al., 2000). However, such assays require ongoing infection, and the results can be greatly affected by sampling methods. In addition, RT-PCR requires diagnostic facilities capable of performing molecular biology techniques, something many state diagnostic laboratories lack.

Thus, what is needed is an improved method to detect an animal exposed to an astrovirus, e.g., TAsV-2.

Summary of the Invention

5 The invention provides a method to identify an animal, e.g., an avian, including but not limited to turkeys, chickens, ostrich, game birds and water fowl such as ducks and geese, or a mammal, e.g., including but not limited to a human, bovine, equine, porcine, feline, canine, caprine and ovine, exposed to turkey astrovirus-2 (TAsV-2). In one embodiment, a physiological fluid sample, such as a
10 serum, bile, or sputum sample, from an animal suspected of being or having been infected with turkey astrovirus-2, is contacted with an antigen of turkey astrovirus-2, and the presence or amount of turkey astrovirus-2 specific antibodies in the sample detected or determined. As used herein, an “antigen” is a sequence in a peptide or polypeptide and optionally highly related sequences, e.g., those having at
15 least 90% amino acid sequence identity, which is specifically bound by antibodies present in physiological fluids of turkey astrovirus-2 infected animals, and is immunogenic, i.e., capable of eliciting the production of specific antibodies in an animal to which the peptide or polypeptide is administered. As used herein, “turkey astrovirus-2” is an astrovirus which is associated with diarrhea or enteritis in young
20 turkey flocks (< about 4 weeks in age) and/or PEMS, and causes diarrhea, growth depression and/or a reduction in thymic mass in susceptible animals, has at least about 80%, and preferably at least about 90%, nucleic acid sequence identity with at least 200, preferably at least 1,000, and up to at least 2,000 to 3,000 or more, e.g., up to about 8,000, contiguous nucleotides of SEQ ID NO:6, an open reading frame
25 thereof, or the complement thereof, encodes a protein that shares at least about 60%, preferably at least 80%, and more preferably at least about 90%, contiguous amino acid sequence identity with at least 150, preferably at least 500, contiguous amino acid residues of, and up to the full-length of, one of SEQ ID NO:7 (encoded by SEQ ID NO:26), SEQ ID NO:8 (encoded by SEQ ID NO:27), or SEQ ID NO:9 (encoded
30 by SEQ ID NO:28), and/or binds antibodies specific for residues 32 to 47, 194 to 221 or 676 to 691 of SEQ ID NO:9, or the corresponding residues in a capsid

protein encoded by any one of SEQ ID NOs:10-14. In one embodiment, turkey
astrovirus-2 is an astrovirus which is associated with PEMS, causes diarrhea,
growth depression and/or a reduction in thymic mass in susceptible animals, and has
at least 60% amino acid sequence identity to SEQ ID NO:9 and optionally binds
5 antibodies specific for residues 32-47, 194-221 or 676-691 or SEQ ID NO:9.

The antigen employed in the method may be an isolated antigen, e.g., one
which is separated from at least one contaminant with which it is ordinarily
associated in its source, as a result of a process that removes the contaminant,
thereby increasing the percent of the antigen. For instance, the antigen may be
10 isolated from virus, an *in vitro* transcription/translation mixture or recombinant cells
which express the antigen. In another embodiment, the sample is contacted with
recombinant cells which express the antigen, or a lysate thereof. In one
embodiment, the recombinant cells are live cells. In another embodiment, the
recombinant cells are fixed, e.g., using paraformaldehyde, formalin, methanol,
15 methanol:acetone or ethanol. The recombinant cells may be prepared by
introducing, e.g., by transfection or infection, to a host cell, for instance, a
prokaryotic or eukaryotic host cell, an expression vector comprising an expression
cassette which encodes an antigen of turkey astrovirus-2. In one embodiment, the
expression cassette encodes a turkey astrovirus-2 capsid protein or an antigenic
20 portion thereof. The expression vector may encode a fusion protein, e.g., a fusion of
a capsid protein or an antigenic portion thereof and another peptide or polypeptide,
such as one which anchors the capsid on the outside of the cell membrane of the
recombinant host cell, is a secretory sequence or is a purification tag. In one
embodiment, the presence of turkey astrovirus-2 specific antibodies in physiological
25 fluid is detected by immunofluorescence or immunohistochemistry, e.g., using an
antibody comprising a label, such as a fluorescent molecule or enzyme, which binds
turkey antibodies.

As described herein, a recombinant baculovirus (rAcNPV-TAstV-2), which
expresses the capsid (outer coat) gene of turkey astrovirus-2 when replicating in
30 insect cells, was prepared. The rAcNPV-TAstV-2 infected cells expressed levels of
turkey astrovirus-2 capsid protein (primarily in the cytoplasm of infected cells),

which were readily and reproducibly detected using fluorescent antibody or colorimetric techniques. Using monolayers of infected cells, the presence of turkey astrovirus-2 antibodies in serum from experimentally infected turkeys as well as serum isolated from commercial turkey flocks was detected. The present
5 serological assay which employs monolayers of infected cells to detect turkey astrovirus-2 antibodies provided more reproducible results than an assay which employed a crude (unpurified) *in vitro* transcription/translation mixture for turkey astrovirus-2 capsid to detect an immune response in turkeys experimentally infected with turkey astrovirus-2. Antibodies to turkey astrovirus-2 (for instance,
10 convalescent serum or isolated anti-turkey astrovirus capsid peptide antibodies) may serve as a positive control and non-specific turkey antiserum as a negative control. As turkeys are commonly infected with astrovirus within 2 weeks of birth, physiological fluid may be tested for antibodies to astrovirus, and optionally other pathogens, at 3-4 weeks after birth, and monitored at later times as well, since
15 reinfection is common. In addition, as other animals, for instance chickens or cows, may be carriers of astrovirus, the assay may be employed with samples from animals that are not susceptible to turkey astrovirus-2 associated disease.

The assay utilizes tools and techniques which are widely used in state diagnostic facilities, allows for more routine surveillance of flocks, and does not
20 require the presence of virus. Moreover, the assay can be performed in a 96-well plate format for large-scale testing and the results are available within several hours. This is critical when decisions on condemnation must be made quickly. The assay is the only specific serologic test available for turkey astrovirus, and is likely more sensitive than a RT-PCR assay (Koci et al., 2000) and more specific and sensitive
25 than a commonly used non-astrovirus-specific test (Purdue Diagnostic Laboratory), which requires the isolation of intestines from suspicious birds, is labor intensive, and cannot rapidly be performed at many facilities.

Thus, the invention provides a method to detect or determine exposure of an animal to turkey astrovirus-2. The method includes providing one or more
30 physiological fluid samples from one or more animals suspected of being exposed to turkey astrovirus-2. The one or more samples is contacted with an antigen of turkey

astrovirus-2 and then it is detected or determined whether the one or more samples comprise antibodies specific for turkey astrovirus-2, thereby detecting or determining whether the animal was exposed to turkey astrovirus-2.

Also provided is a method to detect antibodies to turkey astrovirus-2 in a
5 physiological fluid sample from an animal. The method includes contacting one or more physiological fluid samples from one or more animals with an antigen of turkey astrovirus-2, and detecting or determining the presence or amount of antibodies specific for turkey astrovirus-2 in the one or more samples.

The invention also provides an isolated nucleic acid molecule
10 (polynucleotide) encoding at least one turkey astrovirus-2 protein or a portion thereof, or the complement of the nucleic acid molecule, wherein the nucleic acid molecule is not SEQ ID NO:6 or the complement thereof. In one embodiment, the isolated nucleic acid molecule comprises any one of SEQ ID NOs: 10-18 and 20-25, or the complement thereof, or encodes a protein or a portion thereof having
15 substantially the same activity as a corresponding polypeptide encoded by one of SEQ ID NOs:10-18 and 20-25. As used herein, "substantially the same activity" includes an activity that is about 10%, 30%, 50%, 90%, e.g., up to 100% or more, the activity of the corresponding full-length polypeptide. In one embodiment, the isolated nucleic acid molecule encodes a polypeptide which is substantially the
20 same as, e.g., having at least 60% or more, e.g., 80%, 90%, 92%, 95%, 97% or 99%, contiguous amino acid sequence identity to, a polypeptide encoded by one of SEQ ID NOs:10-18 and 20-25 but which polypeptide is not any one of SEQ ID NOs:7-9. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence which is substantially the same as, e.g., having at least 80% or 90%, or
25 more, contiguous nucleic acid sequence identity to, one of SEQ ID NOs:10-18 and 20-25, or the complement thereof, but which is not SEQ ID NO:6 or the complement thereof, and, in one embodiment, also encodes a polypeptide having at least 60%, e.g., 80%, 90%, 92%, 95%, 97% or 99%, contiguous amino acid sequence identity to a polypeptide encoded by one of SEQ ID NOs:10-18 and 20-25
30 but which is not any one of SEQ ID NOs:7-9. In one embodiment, the isolated and/or purified nucleic acid molecule encodes a polypeptide with one or more, for

instance, 2, 5, 10, 15, 20 or more, conservative amino acids substitutions relative to a polypeptide encoded by one of SEQ ID NOs:10-18 and 20-25. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine and tryptophan; a group of amino acids having basic side chains is lysine, arginine and histidine; and a group of amino acids having sulfur-containing side chain is cysteine and methionine. Preferred conservative amino acid substitution groups are: valine-leucine-isoleucine; phenylalanine-tyrosine; lysine-arginine; alanine-valine; glutamic-aspartic; and asparagine-glutamine. The nucleic acid molecule of the invention may be employed to express turkey astrovirus-2 proteins, to prepare chimeric genes, e.g., with other viral genes, and/or to prepare recombinant virus. Thus, the invention also provides vectors, isolated polypeptides, recombinant virus, and host cells contacted with the nucleic acid molecules, vectors or recombinant virus of the invention.

Further provided is a kit for the diagnosis of turkey astrovirus-2 infection. In one embodiment, the kit comprises a substrate and isolated antigen of turkey astrovirus-2, e.g., one encoded by SEQ ID NO:6. In another embodiment, the kit comprises a substrate and recombinant cells which express an antigen of turkey astrovirus-2, e.g., a capsid protein or an antigenic portion thereof. Optionally, the kit includes a positive control, a negative control, diluent, an anti-avian antibody comprising a label, or any combination thereof. In one embodiment, the recombinant cells are fixed in the wells of a tissue culture plate, e.g., a 96-well plate.

Further provided is an immunogenic composition comprising an effective (immunogenic) amount of an antigen of turkey astrovirus-2, e.g., capsid protein. In particular, the antigen may be isolated from recombinant cells which express the antigen. In another embodiment, the immunogenic composition comprises recombinant cells which express an immunogenic amount of the antigen. In one embodiment, the antigen is the capsid protein, and when expressed in a recombinant

cell, forms a structure found in an infected cell or virion, i.e., the antigen is full-length and optionally processed to 50 kDa and 30 kDa proteins from a 80 kDa precursor. Optionally, the composition further comprises an effective amount of an immunological adjuvant. The composition, once administered to an animal, e.g., a turkey hen, yields anti-turkey astrovirus-2 antibodies which preferably inhibit or prevent turkey astrovirus-2 infection, enteritis associated with turkey astrovirus-2 infection, and/or PEMS. Such antibodies may be useful for passive immunization. Thus, the invention also provides a method of inducing an immune response in an animal to an antigen of turkey astrovirus-2. The method includes administering to the animal an effective amount of a composition comprising an antigen of turkey astrovirus-2. The composition may be administered orally, mucosally or by subcutaneous or intramuscular injection.

Brief Description of the Figures

- Figure 1. Turkey astrovirus genome structure.
- Figure 2A. Nucleotide sequence of (SEQ ID NO:6), and inferred amino acid sequences encoded by (SEQ ID NOs:7, 8 and 9), one isolate of turkey astrovirus-2.
- Figure 2B. Nucleotide sequence of the capsid gene from exemplary turkey astrovirus-2 isolates (SEQ ID NOs:10-14 and 28).
- Figure 2C. An alignment of six turkey astrovirus-2 capsid genes (SEQ ID NOs:10-14 and 28) and a consensus sequence (SEQ ID NO:25).
- Figure 2D. Nucleotide sequence of ORF1a (SEQ ID NOs:22-24) and ORF1b (SEQ ID NOs:15-18 and 20-21) from exemplary turkey astrovirus-2 isolates.
- Figure 3. TAsV-2 IgG and IgA responses following infection.
- Figure 4. Vectors for TAsV-2 protein expression in insect cells.

Detailed Description of the Invention

Enteric disease is a common problem in young turkeys, resulting in substantial financial losses to the poultry industry. Unfortunately, the effects of enteric disease continue long after clinical recovery and can influence the life

quality and value of the flocks. Many different viruses are known to cause enteric disease including retrovirus, coronavirus, enterovirus, and astrovirus. Astroviruses are involved in enteritis in young turkeys due to infection early in life, e.g., at weeks 2 and 3, and the presence of astrovirus increases the probability that a flock will
5 develop PEMS and suffer from severe mortality, resulting in serious financial losses. Testing for astrovirus has become a common way to monitor young turkey flocks. The outcome of the test can determine the fate of a flock (i.e., if the flock is turkey astrovirus-2-positive at a young age, it will commonly be condemned rather than face the losses). Additionally, environmental testing of poultry houses and
10 water for turkey astrovirus-2 is becoming more common given the stability and potential zoonotic transmission of the virus.

I. Astrovirus Genome Organization and Molecular Characterization

Astroviruses are small RNA viruses that are incredibly stable in the environment and resistant to many commercial disinfectants. Astroviruses contain a
15 single stranded positive sense RNA genome typically 7-8 kb in length (Lukashov et al., 2002). The complete sequence of five human astroviruses (HAstVs) isolates (Jiang et al., 1993; Lewis et al., 1994; Willcocks et al., 1994) (GenBank accession AF141381, AF260508), two turkey isolates (Jonassen et al., 1998; Koci et al., 2000b), avian nephritis virus (ANV) (Imada et al., 2000), a sheep astrovirus
20 (OAstV) (Jonassen et al., 1998), and a mink astrovirus (Englund et al., 2002) are available in GenBank.

The basic organization and replication strategy is conserved among all of the astroviruses sequenced. The astrovirus genome includes a 5' untranslated region (UTR), followed by three open reading frames (ORFs), a 3' UTR, and a poly-A tail
25 (Figure 1). The 3 ORFs are designated ORF1a, ORF1b, and ORF2 (Willcocks et al., 1994). The 5' reading frame, ORF1a, is predicted to encode nonstructural proteins including a viral serine protease likely important in processing and maturation of each of the polyproteins encoded in this first reading frame (Willcocks et al., 1994; Geigenmuller et al., 2002; Gibson et al., 1998; Kiang et al.,
30 2002; Willcocks et al., 1999). This viral protease is similar to chymotrypsin-like proteases of other positive sense RNA viruses, although it differs in that a serine

residue has been substituted for a cysteine in the third catalytic position (Gorbalenya et al., 1989; Matsui & Greenberg, 2001). Alignments of the 3 avian AstVs (AAstVs) ORF1a predicted amino acid (aa) sequences allowed for identification of a putative serine protease. When compared to the mammalian AstV (MAstV) serine protease sequence, the three predicted catalytic residues can be identified and are conserved. For instance, the serine residues do align, as well as many of the residues predicted to be important in substrate binding. There is a one-residue shift of the second catalytic aa (aspartic acid) between the AAstVs and the MAstVs.

Downstream of the serine protease, ORF1a is believed to encode a nuclear localization signal (NLS). This putative NLS is 664 aa from the N-terminus of the ORF1a polyprotein of HAstV1 (Willcocks et al., 1999). The need or function of an NLS in an RNA virus is still unclear, but several investigators described limited nuclear staining for astrovirus antigen (Aroonprasert et al., 1989; Willcocks et al., 1999). A similar motif was identified for ANV, corresponding to aa positions 719-735 (Imada et al., 2000). Similar aa sequences can be found in both turkey astrovirus (TAstVs), but none of the putative AAstV NLSs have been tested experimentally. Examination of HAstV ORF1a identified 4 potential transmembrane helical motifs, a putative bipartite nuclear localization signal (NLS), and a region referred to as the immune response element (IRE) identified by antiserum produced against purified particles (Gibson et al., 1998; Willcocks et al., 1999).

The overall ORF1a sequence similarities between the AAstVs and the MAstVs is quite low ranging from 20-25% nucleotide identity (12-15% amino acid identity). However, it is the presence of astrovirus-like nonstructural motifs that is most important. ORF1a is also the most conserved among the HAstVs, and has been used to define two distinct genogroups (Belliot et al., 1997). This is not the case for the AAstVs sequenced to date. There is a greater relatedness among the HAstVs, and to lesser extent sheep astrovirus (OAstVs), than among AAstVs. This suggests AAstV non-structural proteins are allowed greater flexibility in sequence variation than their mammalian counterparts. This may be related to differences in host range (Schneider & Roossinck, 2001). There is no evidence that the MAstVs cross species

lines (Matsui & Greenberg, 2001). However, based on surveillance studies of chicken and turkey farms, antibodies against ANV were isolated from both chickens and turkeys suggesting either support ANV replication (Nicholas et al., 1988; Cavanagh, 1992). Having greater genetic flexibility may increase the likelihood of replicating in whatever poultry species is available, so long as the overall functional motif is conserved (Schneider & Roossinck, 2001).

The first start codon of ORF1b for the HAstVs is found more than 400 nt inside the reading frame, in a suboptimal position according to Kozak's rules (Matsui & Greenberg, 2001). The ORF1a/ORF1b overlap region contains a heptameric shift sequence (A AAA AAC) and the potential for the formation of a downstream stem-loop and possible pseudoknot that would provide a ribosomal frameshift mechanism (Willcocks et al., 1994; Lewis & Matsui, 1995; Lewis & Matsui, 1996; Imada et al., 2000; Koci et al., 2000b). This mechanism is similar to that used by retroviruses and coronaviruses, however, unlike those viruses, the pseudoknot is not required for the astrovirus frameshift to occur (Lewis & Matsui, 1997). This heptameric sequence, and predicted secondary structure has been identified in all three avian AstVs. It is believed that this frameshift structure allows for the translation of ORF1a and ORF1b to occur as one polyprotein that is then cleaved into functional subunits. Analysis of ORF1b, indicates that it encodes for an RNA dependent RNA polymerase (RdRp) (Poch et al., 1989; Ishihama & Barbier, 1994; Lewis et al., 1994; Marczinke et al., 1994). This region of the astrovirus genome is the most conserved between the MAstVs and the AAstVs, as well as among the AAstVs. The RNA-dependent RNA polymerase is liberated from the polyprotein by the serine protease from ORF1a (Lewis et al., 1998).

The final ORF, ORF2, encodes the viral structural protein (Carter et al., 1996). This region encodes a precursor protein with a mass between 75 kilodalton (kDa) and 90 kDa (depending on species) (Jonassen et al., 2001; Wang et al., 2001). The intracellular processing of this sole structural precursor is not well understood (Bass et al., 2000; Menandez et al., 2002). ORF2 is transcribed into a subgenomic message, which is one of the key features, along with the ribosomal frameshift in ORF1a, which led to the classification of astroviruses into their own family.

The lengths of each of these features vary between species and serotypes. For instance, among the three AAstVs there is some variation in the overall lengths of the genomes and their respective internal components (Table 1; Figure 1). In addition to variation in ORF lengths, there are also differences in the expression strategies for ORF2. Most MAstVs (with the exception of HAstV-8) have an overlap of approximately 8 nucleotides (nt) between the stop codon of ORF1b and the start codon of ORF2, which is in the same reading frame as ORF1a. However, the AAstVs deviate from this somewhat in their genome structure. The start codon for ORF2 of ANV is 19 nt downstream of the stop codon of ORF1b, though ORF2 is still in the same frame as ORF1a (Figure 1). The space between the ORF1b stop codon and ORF2 start site for both TAstVs is 18 nt (Figure 1), placing the TAstV ORF2 in the same frame as ORF1b (Figure 1). There are also some differences among the AAstVs toward the end of the genome. Sequence analysis of the last 19 nt of ORF2 and adjacent 3' UTR by (Jonassen et al., 1998; Jonassen et al., 2001) described a conserved sequence and predicted secondary structure present in all astrovirus isolates sequenced, except for TAstV-2 (Figure 1). This conserved motif is also present in infectious bronchitis virus (a coronavirus) and equine rhinovirus type 2 (a picornavirus) (Jonassen et al., 1998).

Table 1. Comparison of the nucleotide lengths of the AAstV genome regions.

Avian astrovirus	Number of nucleotides in					Total ^a
	5' UTR	ORF 1a	ORF 1b	ORF 2	3' UTR	
ANV	14	3012	1527	2052	305	6927
TAstV-2	21	3378	1584	2175	196	7325
TAstV-1	11	3300	1539	2016	130	7003nt

^aexcluding the poly-A tail

II. Immune Response to Astrovirus Infection

Both B cells and T cells respond to human astrovirus infection, and virus-neutralizing antibodies are considered key to astrovirus resistance in humans. Human volunteer studies demonstrated that those with pre-existing antibody titers did not show signs of astrovirus disease. The protective role of virus-specific antibodies has also been demonstrated therapeutically as intravenous immunoglobulin therapy has been used to treat persistent astrovirus infections in immune compromised patients. Astrovirus infections are typically associated with immature or infirmed immune systems. In these hosts, the role of humoral and cellular immunity is hindered or non-existent, however, astroviruses seldom establish persistent infections.

Serial dilutions of sera and bile isolated at 11 and 21 days post-infection of turkey poultts with turkey astrovirus-2 were incubated with a crude *in vitro* transcription/translation mixture of turkey astrovirus-2 capsid prepared using pcDNA3.1⁺ as the expression vector (pcDNA3.1⁺/TAsVcap10). In particular, microtiter plates with the capsid mixture were contacted with sera or bile, and IgG and IgA detected using alkaline phosphatase conjugated goat anti-chicken IgG or IgA (Figure 3; ELISA titers are reported as the reciprocal of the dilution factor). In contrast to humans, there is little evidence of an adaptive immune response following astrovirus infection in otherwise healthy turkeys (Figure 3). The lack of acquired immunity to turkey astrovirus-2 infection suggests the turkey model may reflect the host response in a non-competent immune host.

III. Serological Assay to Detect TAsV-2 Infection

The present invention provides a serologic test for astrovirus infection, e.g., turkey astrovirus-2. This test is specific to astrovirus, easy to adapt if the virus evolves, expandable to different types of enteric viruses, rapid, and can be used by any diagnostic laboratory such as those performing immunofluorescence or immunohistochemistry. Finally, because the test detects antibodies, there is no risk of missing the window of opportunity needed to detect virus using nucleic acid-based amplification strategies.

The invention employs an antigen of turkey astrovirus-2. In one embodiment, the antigen is provided in the form of recombinant cells transformed

with an expression vector encoding one or more antigens of turkey astrovirus-2. In another embodiment, the antigen is provided as isolated turkey astrovirus-2 antigen, e.g., antigen isolated from virus, an *in vitro* transcription/translation reaction or a recombinant cell comprising an expression vector encoding one more turkey
5 astrovirus-2 antigens.

A. Preparation of Expression Cassettes and Recombinant Host Cells

Sources of nucleotide sequences from which the present nucleic acid molecules encoding an antigen of turkey astrovirus-2, or the nucleic acid complement thereof, include RNA or cDNA from any isolate of turkey astrovirus-2, e.g., from physiological fluid or tissue of an animal infected with turkey astrovirus-
10 2, preferably an infected avian. Other sources of the DNA molecules of the invention include cDNA libraries derived from any turkey astrovirus-2-infected cellular source.

A nucleic acid molecule encoding an antigen of turkey astrovirus-2 can be identified and isolated using standard methods, as described by Sambrook et al.,
15 (1989). For example, reverse-transcriptase PCR (RT-PCR) can be employed to isolate and clone turkey astrovirus-2 cDNAs. A primer which is complementary to the RNA encoding a turkey astrovirus-2, and preferably hybridizes to the 3' two-thirds of the RNA can be employed as a primer in a reverse transcriptase reaction to
20 prepare first-strand cDNAs from isolated RNA which contains RNA sequences of interest, e.g., total RNA isolated from an infected avian tissue. RNA can be isolated by methods known to the art, e.g., using TRIZOL™ reagent (Invitrogen). Resultant first-strand cDNAs are then amplified in PCR reactions.

“Polymerase chain reaction” or “PCR” refers to a procedure or technique in
25 which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers comprising at least 7-8 nucleotides. These primers will be identical or similar in sequence to opposite strands of the template to be amplified.
30 PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage

or plasmid sequences, and the like. See generally Mullis et al. (1987); Erlich, (1989). Thus, PCR-based cloning approaches rely upon conserved sequences deduced from alignments of related gene or polypeptide sequences.

5 Primers are made to correspond to highly conserved regions of polypeptides or nucleotide sequences which were identified and compared to generate the primers, e.g., by a sequence comparison of other astrovirus genes. One primer is prepared which is predicted to anneal to the antisense strand, and another primer prepared which is predicted to anneal to the sense strand, of a DNA molecule which encodes an antigen of turkey astrovirus-2.

10 The products of each PCR reaction are separated via an agarose gel and all consistently amplified products are gel-purified and cloned directly into a suitable vector, such as a known plasmid vector. The resultant plasmids are subjected to restriction endonuclease and dideoxy sequencing of double-stranded plasmid DNAs. Alternatively, the gel-purified fragment can be directly sequenced.

15 As used herein, the terms "isolated and/or purified" refer to *in vitro* isolation of a nucleic acid molecule or polypeptide molecule from its natural cellular environment, and from association with other components of the cell, such as nucleic acid or polypeptide. For example, "isolated turkey astrovirus-2 nucleic acid" is RNA or DNA containing greater than 9, preferably 36, and more preferably
20 45 or more, sequential nucleotide bases that encode at least a portion of a protein of turkey astrovirus-2, or a RNA or DNA complementary thereto, that is complementary or hybridizes, respectively, to RNA or DNA encoding a protein of turkey astrovirus-2 and remains stably bound under stringent conditions, as defined by methods well known in the art, e.g., in Sambrook et al., supra. Thus, the RNA or
25 DNA is "isolated" in that it is free from at least one contaminating nucleic acid with which it is normally associated in the natural source of the RNA or DNA and is preferably substantially free of any other RNA or DNA. The phrase "free from at least one contaminating source nucleic acid with which it is normally associated" includes the case where the nucleic acid is reintroduced into the source or natural
30 cell but is in a different location or is otherwise flanked by nucleic acid sequences not normally found in the source. An example of isolated turkey astrovirus-2

nucleic acid is RNA or DNA that shares at least about 80%, and more preferably at least about 90%, nucleic acid sequence identity with at least 15 contiguous nucleotides of SEQ ID NO:6, any one of SEQ ID NOs:10-18 and 20-28, or the complement thereof, or at least about 80% nucleic acid sequence identity with at least 200 and up to 1,500, e.g., up to 3,000, or more nucleotides of SEQ ID NO:6, an open reading frame therein, any one of SEQ ID NOs:10-18 and 20-28, or the complement thereof, or encodes a protein that shares at least about 60%, preferably at least about 80%, and more preferably at least about 90%, amino acid sequence identity with at least 15 contiguous residues of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or the protein encoded by any one of SEQ ID NOs:10-18 and 20-25, or at least about 60%, preferably at least about 80%, and more preferably at least about 90%, amino acid sequence identity with at least 150 and up to about 500 amino acids or more of, for instance, SEQ ID NO:9 or a protein encoded by any one of SEQ ID NOs:10-14.

As used herein, the term “recombinant nucleic acid” or “recombinant RNA or DNA sequence or segment” refers to a nucleic acid, e.g., to DNA, that has been derived or isolated from any appropriate viral or cellular source, that may be subsequently chemically altered *in vitro*, so that its sequence is not naturally occurring, or corresponds to naturally occurring sequences that are not positioned as they would be positioned in a genome which has not been transformed with exogenous DNA. An example of DNA “derived” from a source, would be a DNA sequence that is identified as a useful fragment within a given organism, and which is then chemically synthesized in essentially pure form. An example of such DNA “isolated” from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering. Therefore, “recombinant DNA” includes completely synthetic DNA sequences, semi-synthetic DNA sequences, DNA sequences isolated from biological sources, and DNA sequences derived from RNA, as well as mixtures thereof.

As used herein, the term “derived” with respect to a RNA molecule means that the RNA molecule has complementary sequence identity to a particular DNA molecule.

5 Nucleic acid molecules encoding amino acid sequence variants of an antigen of turkey astrovirus-2 are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants or serotypes) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, directed evolution, and cassette mutagenesis of an earlier prepared variant or a non-variant
10 version of an antigen of turkey astrovirus-2.

In one embodiment, the nucleic acid sequence for an antigen of turkey astrovirus-2 is altered to encode a polypeptide or peptide with one or more amino acid substitutions relative to the polypeptide or peptide encoded by the unaltered nucleic acid sequence. Preferably, the altered nucleic acid sequence encodes a
15 polypeptide or peptide that is antigenic, e.g., binds antibodies specific for turkey astrovirus-2. Conservative amino acid substitutions are preferred--that is, for example, aspartic-glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

20 Conservative substitutions within the scope of the invention include those shown in Table 2 under the heading of exemplary substitutions. More preferred substitutions are under the heading of preferred substitutions. After the substitutions are introduced, the variants are screened for activity, for binding to antibodies specific for turkey astrovirus-2.

TABLE 2

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; lys; arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	asn; gln; lys; arg	Arg
Ile (I)	leu; val; met; ala; phe norleucine	Leu
Leu (L)	norleucine; ile; val; met; ala; phe	Ile
Lys (K)	arg; gln; asn	Arg
Met (M)	leu; phe; ile	Leu
Phe (F)	leu; val; ile; ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	trp; phe; thr; ser	Phe
Val (V)	ile; leu; met; phe; ala; norleucine	Leu

- 5 Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into
- 10 groups based on common side-chain properties:
- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
 - (2) neutral hydrophilic: cys, ser, thr;
 - (3) acidic: asp, glu;
 - (4) basic: asn, gln, his, lys, arg;

- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic; trp, tyr, phe.

The invention also envisions polypeptide or peptide variants with non-conservative substitutions. Non-conservative substitutions entail exchanging a
5 member of one of the classes described above for another.

Once a particular nucleic acid sequence or molecule is selected, it is introduced into an expression cassette. Expression cassettes may be circular or linear, double-stranded or single-stranded. A DNA sequence which encodes an RNA sequence that is substantially complementary to a RNA sequence encoding an
10 antigen of turkey astrovirus-2 is typically a "sense" DNA sequence cloned into a cassette in the opposite orientation (i.e., 3' to 5' rather than 5' to 3'). Generally, expression cassette is in the form of chimeric DNA that contains a coding region flanked by control sequences for the expression of the DNA sequence, or otherwise serve a regulatory or a structural function. "Chimeric" means that a vector
15 comprises DNA from at least two different species or sources, or comprises DNA from the same species, which is linked or associated in a manner which does not occur in the "native" or wild type of the species (immature). Control sequences are DNA sequences for the expression of an operably linked coding sequence in a particular host cell organism. The control sequences that are suitable for
20 prokaryotic cells, for example, include a promoter, and optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. For example, the expression vector may comprise an expression cassette comprising a promoter that is active in eukaryotic cells operably linked to a coding sequence. Exemplary promoters in
25 eukaryotes include viral promoters such as a CMV promoter, a SV40 late promoter, retroviral LTRs (long terminal repeat elements), and a baculovirus promoter, although many other promoter elements for eukaryotic, as well as prokaryotic cells, which are well known to the art, may be employed in the practice of the invention.

"Operably linked" is defined to mean that nucleic acids are placed in a
30 functional relationship with each other. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a

ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation; DNA for a presequence or secretory leader is operably linked to DNA for a peptide or polypeptide if it is expressed as a preprotein that participates in the secretion of the peptide or polypeptide; or a DNA for an epitope, purification tag or membrane-spanning domain is operably linked to DNA for a peptide or polypeptide if it is expressed as a fusion protein and facilitates detection, purification or localization of that fusion. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, epitope, purification tag or other domain, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is often accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

Elements such as introns, enhancers, polyadenylation sequences and the like, may or may not be necessary for the function of the DNA, but may provide improved expression of the DNA sequence by affecting transcription, stability of the mRNA, or the like. Such elements may be included in the expression cassette as desired to obtain the optimal performance of the expression cassette in the cell.

The recombinant DNA containing the expression cassette to be introduced into the cells may also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of transformed cells from the population of cells sought to be transformed. Alternatively, the selectable marker may be carried on a separate piece of DNA and used in a co-transformation procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are well known in the art and include, for example, antibiotic and herbicide-resistance genes, such as *neo*, *hpt*, *dhfr*, *bar*, *aroA*, *dapA* and the like. See also, the genes listed on Table 1 of Lundquist et al. (U.S. Patent No. 5,848,956).

Reporter genes are used for identifying potentially transformed cells and for evaluating the functionality of regulatory sequences. Reporter genes which encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene which is not present in or expressed by the recipient organism or tissue and

which encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Exemplary reporter genes include the chloramphenicol acetyl transferase gene (*cat*) from Tn9 of *E. coli*, the beta-glucuronidase gene (*gus*) of the *uidA* locus of *E. coli*, and the luciferase gene, e.g.,
5 from the firefly *Photinus pyralis*. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells.

The general methods for constructing recombinant DNA which can transform target cells are well known to those skilled in the art, and the same compositions and methods of construction may be utilized to produce the DNA
10 useful herein. For example, Sambrook et al. (1989) provides suitable methods of construction.

A vector comprising a recombinant DNA, for instance, a vector comprising an expression cassette of the invention, can be readily introduced, e.g., transfected or via infection, into host cells, e.g., mammalian, bacterial, e.g., *E. coli* or
15 *Salmonella*, fungal, yeast or insect cells, by any procedure useful for the introduction of nucleic acid into a particular cell, e.g., physical or biological methods, to yield a recombinant cell having the recombinant DNA. The host cells of the present invention are typically produced by transfection or infection with a DNA sequence in a plasmid expression vector, a viral expression vector, or as an isolated
20 linear DNA sequence. The host cell is preferably of insect origin, but cell lines or host cells of non-insect origin may be employed, including avian, plant, mammalian, yeast, fungal or bacterial sources.

Physical methods to introduce a recombinant DNA into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection,
25 electroporation, and the like. Biological methods to introduce the DNA of interest into a host cell include the use of DNA and RNA viral vectors. Viral vectors can be derived from poxviruses, herpes simplex virus I, retroviruses, baculoviruses, adenoviruses and adeno-associated viruses, and the like.

To confirm the presence of the recombinant DNA sequence in the host cell,
30 a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and

Northern blotting, RT-PCR and PCR; or "biochemical" assays, such as detecting the presence or absence of a turkey astrovirus-2 polypeptide, e.g., by immunological means (ELISAs, immunofluorescence, immunohistochemistry, and Western blots).

B. Isolated Antigen

5 Sources of antigen useful in the methods of the invention include turkey astrovirus-2 virions or degradation products thereof, turkey astrovirus-2 peptide or polypeptide products of an *in vitro* reaction such as a chemical synthesis or an *in vitro* transcription/translation mixture, and recombinant cells expressing one or more turkey astrovirus-2 peptides or polypeptides, or antigenic portions thereof. An
10 antigenic "portion" is generally an amino acid sequence of at least about five consecutive amino acids of a particular peptide or polypeptide but less than the sequence of the full-length peptide or polypeptide. Virus may be propagated in eggs and isolated by known methods. Specific viral proteins in the turkey astrovirus-2 viral preparation may be separated by known techniques, yielding isolated turkey
15 astrovirus-2 protein. Alternatively, turkey astrovirus-2 protein may be obtained synthetically, e.g., via chemical synthesis or recombinant means. As used herein, a turkey astrovirus-2 peptide or polypeptide includes turkey astrovirus-2 peptides or polypeptides having one or more modifications, e.g., insertions, deletions or substitutions, which do not substantially alter the binding of the resulting peptide or
20 polypeptide to anti-turkey astrovirus-2 antibodies found in infected animals relative to the binding of the corresponding non-modified (wild-type) peptide or polypeptide to those antibodies.

 Turkey astrovirus-2 peptides or polypeptides can be synthesized *in vitro*, e.g., by the solid phase peptide synthetic method or by recombinant DNA
25 approaches. When a turkey astrovirus-2 polypeptide of the invention is expressed in a recombinant cell, the polypeptide may be purified from other recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as to the turkey astrovirus-2 peptide or polypeptide. For example, the culture medium or lysate can be centrifuged to remove particulate cell debris. The membrane and
30 soluble protein fractions are then separated. The turkey astrovirus-2 polypeptide may then be purified from the soluble protein fraction. Alternatively, the turkey

astrovirus-2 polypeptide may be purified from the insoluble fraction, i.e., refractile bodies (see, for example, U.S. Patent No. 4,518,526), if necessary. Turkey
astrovirus-2 peptide or polypeptide may be purified from contaminant soluble or
membrane proteins and polypeptides by fractionation on immunoaffinity or ion-
5 exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on
silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE;
ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75;,
or ligand affinity chromatography; ultracentrifugation, and the like.

If expressed as a fusion polypeptide, the fusion polypeptide may be purified
10 by methods specific for the non-turkey astrovirus-2 polypeptide portion of the
polypeptide. For example, if the fusion polypeptide is a glutathione-S transferase
(GST) fusion polypeptide, GST 4B beads may be employed to purify the fusion
polypeptide.

Turkey astrovirus-2 polypeptide or a portion thereof, can also be prepared by
15 *in vitro* transcription and translation reactions. A turkey astrovirus-2 polypeptide
expression cassette can be employed to generate turkey astrovirus-2 gene-specific
transcripts which are subsequently translated *in vitro* so as to result in a preparation
of substantially homogenous turkey astrovirus-2 peptide or polypeptide. The
construction of vectors for use *in vitro* transcription/translation reactions, as well as
20 the methodologies for such reactions, are well known to the art.

The solid phase peptide synthetic method is an established and widely used
method to prepare peptides and polypeptides, which is described in the following
references: Stewart et al., 1969; Merrifield, 1963; Meienhofer, 1973; and Bavaay
and Merrifield, 1980). These polypeptides or peptides can be further purified by
25 fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation;
reverse phase HPLC; chromatography on silica or on an anion-exchange resin such
as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel
filtration using, for example, Sephadex G-75; or ligand affinity chromatography.

Once isolated and characterized, derivatives, e.g., chemically derived
30 derivatives, of a given turkey astrovirus-2 polypeptide or peptide can be readily
prepared. For example, amides of the turkey astrovirus-2 polypeptide, peptide or

variants thereof of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the peptide from a solid support with an appropriate amine, or to cleave in the
5 presence of an alcohol, yielding an ester, followed by aminolysis with the desired amine.

Salts of carboxyl groups of a polypeptide or peptide of the invention may be prepared in the usual manner by contacting the polypeptide or peptide with one or more equivalents of a desired base such as, for example, a metallic hydroxide base,
10 e.g., sodium hydroxide; a metal carbonate or bicarbonate base such as, for example, sodium carbonate or sodium bicarbonate; or an amine base such as, for example, triethylamine, triethanolamine, and the like.

N-acyl derivatives of an amino group of the polypeptide or peptide of the invention may be prepared by utilizing an N-acyl protected amino acid for the final
15 condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy peptide or peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired.

20 Formyl-methionine, pyroglutamine and trimethyl-alanine may be substituted at the N-terminal residue of the polypeptide or peptide. Other amino-terminal modifications include aminooxypentane modifications (see Simmons et al., 1997).

In addition, the amino acid sequence of the polypeptide or peptide can be modified as described above and including substitutions which utilize the D rather
25 than L form, as well as other well known amino acid analogs.

Acid addition salts of the polypeptide or peptide or of amino residues of the polypeptide or peptide may be prepared by contacting the polypeptide or peptide, or amine thereof with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the

5 polypeptides or peptides may also be prepared by any of the usual methods known in the art.

C. Exemplary Assays

The present invention relates to assays for use in veterinary medicine. In one embodiment, turkey astrovirus-2 antigen is employed to detect or determine the
10 presence or amount of anti-turkey astrovirus-2 antibodies in an animal. In particular, the use of turkey astrovirus-2 antigen with physiological fluid from an animal can detect whether the animal has been exposed to turkey astrovirus-2, i.e., due to the presence of antibodies in physiological fluid which bind to a turkey astrovirus-2 polypeptide or peptide, and so is at risk of infecting other animals or succumbing to
15 viral induced pathology. Animals which have anti-turkey astrovirus-2 antibodies may be at risk of developing or have PEMS, a convalescent animal or a carrier of turkey astrovirus-2.

The invention thus provides a method for detecting or determining the presence antibodies which are specific for turkey astrovirus-2 in an animal
20 physiological fluid sample. The method comprises contacting an amount of antigen (native or recombinant) of turkey astrovirus-2, with the physiological sample which comprises antibodies suspected of specifically reacting with turkey astrovirus-2, for a sufficient time to form binary complexes between at least a portion of the antibodies and the antigen. Then the presence or amount of the complexes is
25 detected or determined. In one embodiment, recombinant cells transfected or infected with nucleic acid encoding an antigen of turkey astrovirus-2, such as a lysate of those cells, can be employed as the antigenic material that is contacted with the physiological sample to be tested. The invention also provides kits useful to detect or determine the presence of antibodies that specifically react with an
30 infectious agent which is associated with PEMS. Such a kit may comprise packaging, containing, separately packaged: (a) cells transfected with turkey

astrovirus-2 nucleic acid or infected with turkey astrovirus-2, e.g., cells which are fixed; and (b) a solid phase capable of immobilizing the cells. Such a kit may also comprise packaging, containing, separately packaged: (a) isolated turkey astrovirus-2 antigen, e.g., capsid protein from cells transfected with turkey astrovirus-2 nucleic acid or infected with turkey astrovirus-2; and (b) a solid phase capable of immobilizing the antigen.

Exemplary means for detecting and/or quantitating turkey astrovirus-2 antibody in body fluids, including supernatants from homogenized tissue samples or tissue sample lysates, include affinity chromatography, Western blot analysis, immunoprecipitation analysis, agglutination, hemagglutination as well as immunoassays including but not limited to immunohistochemistry, ELISAs (enzyme-linked immunosorbent assays), RIA (radioimmunoassay), IFA (immunofluorescent assays), competitive EIA or dual antibody sandwich assays. Immunoassays are a preferred means to detect turkey astrovirus-2. The assays can be performed using standard protocols such as those described by Magnarelli et al., 1984; Craft et al., 1984; Enguall et al., 1971; and Russell et al., 1984.

Representative immunoassays involve the use and/or detection of at least one antibody specific for turkey astrovirus-2 in the body fluid of an animal. The moiety employed to detect the antigen bound antibodies may be detectable, e.g., a moiety which is labeled or otherwise capable of detection, e.g., using a molecule which interacts with the moiety. Unlabeled antibodies may be employed in agglutination; labeled antibodies or other binding molecules may be employed in a wide variety of assays, which can employ a wide variety of labels. Suitable detection means include the use of labels such as radionuclides, enzymes, fluorescent molecules, chemiluminescent molecules, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes, beads, e.g., gold beads, and the like. Such labeled reagents may be used in a variety of well known assays. See for example, U.S. Patent Nos. 3,766,162, 3,791,932, 3,817,837, and 4,233,402. For instance, the detectable moiety may allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry, radiometric measurement, or the like. Examples of detectable moieties include

fluorescein and rhodamine (for fluorescence microscopy or other fluorometric techniques), horseradish peroxidase (for either light or electron microscopy and biochemical detection), biotin-streptavidin (for light or electron microscopy), alkaline phosphatase (for biochemical detection by color change), and luciferase
5 (for luminescence detection by a luminometer or fluorometric techniques). The detection methods and moieties used can be selected, for example, from the list above, or other suitable examples by the standard criteria applied to such selections (Harlow and Lane, 1988; herein incorporated by reference).

In one embodiment, an assay of the present invention can be constructed by
10 coating on a surface (i.e., a substrate such as solid support), for example, a plastic bead, a microtitration plate, a membrane (e.g., nitrocellulose membrane) or an inert particle, for example, bentonite, polystyrene or latex, an antigen such as turkey astrovirus-2 peptide or polypeptide (natural, recombinant or synthetic), or a recombinant host cell expressing one or more turkey astrovirus-2 peptides or
15 polypeptides. The antigen is contacted with serum or other physiological fluid taken from an animal suspected of being exposed to turkey astrovirus-2 or having a turkey astrovirus-2 infection. Following removal of the physiological fluid, any antibody bound to antigen can be detected, for instance, by reacting the binary antibody-antigen complexes with a moiety that binds the antibody, the antigen, or the
20 complex. In one embodiment, the moiety comprises a label (detectable molecule) or binds to a detectable molecule. For example, the moiety may be an antibody comprising a label or a binding site for a detectable molecule. Generally, the secondary antibody is selected for its ability to react with multiple sites on the primary antibody.

25 In one embodiment, a sample from a test subject is reacted with the antigen bound to a substrate (antigen/antibody complex) (e.g., 96 well plate), and excess sample is washed from away. A labeled monoclonal antibody is then reacted with the previously reacted antigen/antibody complex. The amount of inhibition of monoclonal antibody binding is measured relative to a control. The degree of
30 monoclonal antibody inhibition is a very specific test for a particular variety or strain since it is based on monoclonal antibody binding specificity.

In another embodiment, a sample suspected of comprising antibodies to turkey astrovirus-2 is contacted with a surface and the antigen added to the sample. In one embodiment, the sample is covalently linked to the surface. Following removal of any unbound antigen, antigen bound to the sample can be detected, for instance, by reacting the binary antibody-antigen complexes with a moiety that binds the antibody, antigen or the complex. In one embodiment, the moiety comprises a label or binds to a detectable molecule.

In yet another embodiment, the sample and antigen are mixed, and complexes detected, for instance, by reacting the binary antibody-antigen complexes with a moiety that binds the antibody, the antigen, or the complex, e.g., a moiety which is attached to a support.

A micro-agglutination test can also be used to detect the presence of antibodies to avian astroviruses. Latex beads (or red blood cells) are coated with the antigen and mixed with a sample, such that antibodies in the sample that are specifically reactive with the antigen cross-link with the antigen, causing agglutination. The agglutinated antigen-antibody complexes form a precipitate, visible to the naked eye or capable of being detected by a spectrophotometer. In a modification of the above test, antibodies specifically reactive with the antigen can be bound, the beads and the antigen in the sample thereby detected.

IV. Dosages, Formulations and Routes of Administration of the Host Cells and Polypeptides of the Invention

In another embodiment of the invention, turkey astrovirus-2 antigen is employed to elicit a humoral response, e.g., a protective response, in an animal. The antigen of the invention can thus be used in an immunogenic composition comprising an effective amount of the antigen and optionally a pharmaceutically acceptable carrier. The immunogenic composition may include the antigen, or a recombinant host cell which expresses the antigen, e.g., an insect cell or *E. coli* which expresses the antigen. The immunogenic composition can then be used in a method of reducing and or preventing complications of avian astrovirus infection.

Cells which express one or more turkey astrovirus-2 peptides or polypeptides, or isolated polypeptides or peptides, of the invention are preferably

administered to an animal, e.g., a turkey, chicken or bovine, so as to result in an immune response specific for the virus or a related virus. These compounds and compositions can be administered to avians and mammals for veterinary use, such as for use with domestic or farm animals. The recombinant cells compositions may

5 be administered as live, modified-live (attenuated) or inactivated cells, or optionally administered as a combination of attenuated, inactivated, and/or live cells, or in combination with a polypeptide or peptide of the invention, or any combination thereof. Moreover, the administration of more than one immunogenic agent of the invention to an animal may occur simultaneously or at different times. The cells

10 may be inactivated by agents including, but not limited to, formalin, phenol, ultraviolet radiation, and β -propiolactone. In particular, for administration of polypeptide or peptide of the invention, e.g., subcutaneously, *in ovo*, orally or intramuscularly, to a bird, e.g., turkeys or chickens, the amount administered may be at dosages of at least about 1 μ g to about 10 mg, preferably about 10 μ g to about 1

15 mg, and more preferably about 100 μ g to about 500 μ g, although other dosages may provide beneficial results. For administration of recombinant cells, the amount administered may be at dosages of at least about 10^4 to about 10^7 cells, e.g., cells which may be administered subcutaneously, *in ovo*, orally or intramuscularly, although other dosages may provide beneficial results. Dosages within these ranges

20 can be administered via bolus doses or via a plurality of unit dosage forms, until the desired effects have been obtained. The amount administered will vary depending on various factors including, but not limited to, the specific immunogen chosen, the weight, physical condition and age of the animal, and the route of inoculation. Thus, for peptides and polypeptides, the absolute weight of the polypeptide or

25 peptide included in a given unit dosage form of vaccine can vary widely, and depends upon factors such as the species, age, weight and physical condition of the animal considered for vaccination, as well as the method of administration. Such factors can be readily determined by the veterinarian employing animal models or other test systems which are well known to the art. A unit dose of a polypeptide or

30 peptide vaccine is preferably administered parenterally, e.g., by subcutaneous or by intramuscular injection.

The polypeptides or peptides of the invention may also be conjugated or linked to an immunogenic protein, such as KLH or albumin, to enhance their immunogenicity. For example, synthetic peptides are coupled to KLH through the C-terminal cysteine of the peptide using the heterobifunctional reagent *N*-γ-maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS; Sigma). Carrier protein [4 mg KLH ml⁻¹ in 100 μl phosphate buffered saline (PBS) pH 7.4] is activated by reaction with GMBS (0.5 mg per 5 μl dimethylformamide) for 1 hour at 25°C under nitrogen gas. The activated protein is separated from excess GMBS by gel filtration on Sephadex G25 (Pharmacia). Column fractions containing the carrier protein (monitored by *A*₂₈₀) are pooled, and added to 4 mg peptide dissolved in an equivalent volume of PBS. The mixture is gassed with nitrogen, and incubated at 25°C for 3 hours with gentle stirring. The progress of the conjugation is monitored colorimetrically from reactivity of free cysteine thiol groups with Ellman's reagent. Coupling is complete when no color change is observed. The carrier-conjugated peptides are stored at -20°C until used.

Preferably, the administration of the antigen to a bird results in an immune response, e.g., the production of antibodies to turkey astrovirus-2, and/or inhibits or prevents PEMS and/or poult enteritis. Both local and systemic administration is contemplated.

Also envisioned is the administration of maternal antibody, which antibody is obtained from a female animal exposed to a recombinant cell, a nucleic acid molecule encoding an antigen of turkey astrovirus-2, or isolated peptide or polypeptide of the invention. For example, a hen is vaccinated with at least one of the immunogenic compositions of the invention. The hen then provides passive immunity to progeny through the transfer of maternal antibody to the embryo. Alternatively, an egg-laying animal may be immunized and the eggs from that animal collected. Antibody is recovered from the eggs and then administered to susceptible animals to provide passive protection.

Typically, immunogenic compositions are prepared for injection or infusion, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection or infusion may also be prepared.

The preparation may also be emulsified. The active ingredient can be mixed with diluents, carriers or excipients which are physiologically acceptable and compatible with the active ingredient(s). Suitable carriers can be positively or negatively charged or neutral avridine-containing liposomes, oil emulsions; live-in-oil; killed-in-oil, water-in-oil; $\text{Al}(\text{OH})_3$; oil emulsion with terpene oils squalene or squalene; or aqueous. Suitable diluents and excipients are, for example, water, saline, PBS, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

Such compositions are conventionally administered parenterally, by injection, for example in birds, either intravenously, intramuscular injection to breast, lung or thigh, subcutaneous injection, wing web injection, or administration via the beak, spraying the animals and their environment, e.g., their housing or yard, or administration in the drinking water or feed. The administration of maternal antibody or recombinant cells is preferably in feed or water, or *in ovo*. Polypeptide or peptide is preferably administered via injection. Formulations which are suitable for other modes of administration include suppositories, cloaca, insufflated powders or solutions, eye drops, nose drops, intranasal aerosols, and oral formulations, e.g., introduced into drinking water. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of alkylcelluloses, mannitol, dextrose, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. Thus, these compositions can take the form of solutions, suspensions, tablets, pills, hard or soft gelatin capsules, sustained-release formulations such as liposomes, gels or hydrogels; or powders, and can contain about 10% to about 95% of active ingredient, preferably at about 25% to about 70%.

One or more suitable unit dosage forms comprising the cell preparations, or polypeptides or peptides of the invention, may optionally be formulated for sustained release. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid

carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

To prepare an immunogenic composition comprising a polypeptide or peptide, the polypeptide or peptide can be isolated as described hereinabove, lyophilized and stabilized. Alternatively, the polypeptide or peptide may be modified so as to result in a derivative polypeptide or peptide, as described above. The polypeptide or peptide antigen may then be adjusted to an appropriate concentration, optionally combined with a suitable carrier and/or suitable vaccine adjuvant, and preferably packaged for use as a vaccine. Suitable adjuvants include, but are not limited to, surfactants, e.g., hexadecylamine, octadecylamine, lysolecithin, di-methyldioctadecylammonium bromide, N,N-dioctadecyl-n'-N-bis(2-hydroxyethyl-propane di-amine), methoxyhexadecyl-glycerol, and pluronic polyols; polyanions, e.g., pyran, dextran sulfate, poly IC, polyacrylic acid, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin, oil emulsions, alum, and mixtures thereof. Finally, the immunogenic product may be incorporated into liposomes for use in a vaccine formulation, or may be conjugated to polysaccharides or other polymers.

A pharmaceutically acceptable carrier can comprise saline or other suitable carriers (Arnon, R. (ed) *Synthetic Vaccines* I. 83 - 92, CRC Press, Inc., Boca Raton, Fla, 1987). An adjuvant can also be a part of the carrier of the vaccine, in which case, it can be selected by standard criteria based on the antigen used, the mode of administration and the subject (Arnon 1987; supra) Methods of administration can be by oral or sublingual means, or by injection, depending on the particular vaccine used and the subject to whom it is administered.

Vaccination schedules and efficacy testing for avians are well known to the art, e.g., see Rimler et al., 1979; Schlink et al., 1987; Wang et al., 1994a; Wang et al., 1994b; Zhang et al., 1994; and Rimler et al., 1981.

The invention will be further described by the following non-limiting examples.

Example I

Materials and Methods

TAstV-2 Propagation. TAstV-2 was isolated and propagated as described in Koci et al. (2000a) and Schultz-Cherry et al. (2001). Briefly, the thymus or intestines from infected turkey poults were homogenized, 0.2 μ m filtered, and
5 inoculated into the yolk sac of 20-day-old specific pathogen-free (SPF) turkey embryos (from a closed flock of Small Beltsville White turkeys housed at Southeast Poultry Research Laboratory). Viral replication in embryo intestines was monitored by *in situ* hybridization at 1, 3, and 5 days post-inoculation (dpi). Virus was harvested at 5 dpi. Intestines were removed, homogenized, 0.2 μ m filtered and
10 centrifuged at 150 x g for 10 minutes. Additionally, embryo intestinal fluid was collected separately, 0.2 μ m filtered and centrifuged at 500 x g for 10 minutes.

RNA Isolation and RT-PCR. Total RNA was isolated from purified virus, embryo intestines, or from tissues excised from experimentally-inoculated or control turkeys using Trizol™ following manufacturer instructions (Invitrogen, Carlsbad
15 CA). RT-PCR was performed as previously described in Koci et al. (2000b).

TAstV-2 Quantitation. Viral load was assessed by developing a TAstV-2-specific competitive quantitative RT-PCR (CQ RT-PCR) system. Briefly, total RNA, isolated from 100 μ l of infectious material, was analyzed by one-step RT-PCR (Qiagen, Valencia CA) in the presence of a competitor RNA (cRNA). The
20 cRNA was generated by modifying a plasmid (pTAstVpol18) which contains nucleotides 2863 to 5296 of the TAstV-2 genome. pTAstVpol 18 was digested with *Sca* I following the manufacturer's instructions (Invitrogen), then two 30 bp randomly generated oligonucleotides were ligated to the cut plasmid to generate a construct with TAstV-2 pol gene with 60 bp of additional sequence (pTAstVpolC).
25 This new construct was then digested with *Sst* I and *Not* I following the manufacturer's instructions (Invitrogen) and ligated into the corresponding sites in pGEM T-Easy vector (Promega, Madison WI). This final construct pTAstVpolCQ, was then used to generate positive sense cRNA using the RNA polymerase SP6 (Roche Molecular, Indianapolis, IN). cRNA was purified, and copy numbers
30 quantitated using spectrophotometry as described in Sambrook et al. (1989). TAstV-2 polymerase gene specific primers, flanking the modified region in pTAstVpolCQ,

were designed (CQ RT-PCR Fwd; CCATGATATGCTACGGGGAT; SEQ ID NO:1) and CQ RT-PCR Rev; GACTCAACATCTGGTAGCCT; SEQ ID NO:2). Sample RNA was added at a uniform concentration to each tube of a serial log dilution of cRNA, and amplified under the following conditions; 50°C for 30 minutes, 95°C for 15 minutes, 30 cycles of 94°C for 30 seconds, 55° C for 30 seconds, 72°C for 30 seconds, and final 72° C extension for 1 minute, using the Qiagen OneStep RT-PCR Kit (Qiagen, Valencia CA) in a total reaction volume of 25 µl. Products were then separated by electrophoresis in an agarose gel and the amplification products visualized with ethidium bromide. The copy numbers of viral RNA in the sample/ml were calculated using Kodak Imaging Software densitometry and plotting against the standard curve of the competitor as previously described in Frieman et al. (1999).

Animals. Two-day-old unvaccinated British United Turkey of America poult (male and female) were obtained from a commercial hatchery. Control and infected poult were housed in separate BL2 containment facilities in individual Horsfall units with HEPA filtered inlet and exhaust air valves. Birds were fed routine turkey starter from the University of Georgia and given free access to clean water. After a brief acclimation period, five-day-old poult were weighed (day 0) and randomly assigned to either a control group or a group infected with astrovirus (n = 60 per group). Poult were orally inoculated with about 10⁶ genomic units of astrovirus in 200 µl total volume, or phosphate buffered saline (PBS) alone. Birds were monitored daily for signs of clinical disease and weighed on 0, 3, 5, 9, and 12 dpi. On days 1, 2, 3, 4, 5, 7, 9, and 12 pi, five random poult per group were euthanized by cervical dislocation and the small intestine, bursa, spleen, pancreas, thymus, liver, kidney, bone marrow, skeletal muscle (breast), feces and blood were collected. All tissues were stored at -70°C or placed in 10% phosphate-buffered formalin. Blood was collected in syringes containing heparin, incubated overnight at 4°C and then separated into red cell, lymphocyte, and plasma fractions using Histopaque 1077 (Sigma Chemicals, St. Louis, MO). The bursa, spleen, and thymus from each group were weighed to the nearest milligram prior to processing.

To perform RT-PCR analysis and virus isolation studies, the individual tissues at each time point were pooled, homogenized, and aliquoted for RNA isolation using Trizol™ or inoculation into 20-day-of-age turkey embryos. The animal experiments were repeated five times with different groups of poult with similar results.

In situ Hybridization. The TAsV-2-specific riboprobe was generated as described in Behling et al. (2002). Briefly, TAsV-2 plasmid p25.5 containing a 1.5 kb segment of the extreme 3' end of the TAsV-2 genome (Koci et al., 2000a) was digested with BamHI and transcribed with T7 RNA polymerase and digoxigenin labeled UTP (Roche Molecular), creating an antisense riboprobe of approximately 1.6 kb in length. Digoxigenin incorporation was verified by dot-blot. *In situ* hybridization was performed according to previously described techniques (e.g., see Brown, 1999). Briefly, tissue sections were deparaffinized with Citrisolv (Fisher Scientific, Norcross GA), digested with 35 µg/ml Proteinase K for 15 minutes at 37°C, and hybridized overnight at 42°C, using approximately 35 ng of digoxigenin-labeled riboprobe per slide in 5X standard sodium citrate (SSC), 50% formamide, 5% modified milk protein (Roche Molecular), 1% N-lauroylsarcosine, and 0.02% SDS. The following day, slides were washed in increasingly stringent solutions, i.e., 2X SSC with 1% SDS for 30 minutes at 50°C, 1X SSC with 0.1% SDS for 30 minutes at 50°, 1X SSC for 15 minutes three times at room temperature, and 0.1 XSSC for 15 minutes at room temperature. After the posthybridization washes, sections were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular) for 2 hr at 37°C and developed with nitroblue tetrazolium and bromocresylindolyl phosphate for 1 to 3 hours. Sections were counter-stained lightly with hematoxylin and coverslipped with Permount for a permanent record. Each group of slides was processed with a positive control tissue consisting of a section of positive embryo intestine, and negative control sections from uninfected poult.

Histopathology. Tissues from control and infected poult were fixed in 10% phosphate buffered formalin overnight, then processed, embedded, sectioned (0.3 µm), and stained with hematoxylin and eosin and examined by light microscopy.

Detection of TAstV-2 Antigen by Immunofluorescence. The distribution of TAstV-2 was monitored using a rabbit polyclonal antibody generated to a peptide sequence in the TAstV-2 capsid protein (K₆₇₆ – R₆₉₁) (ResGen, Carlsbad CA), accession# AAF18464. Briefly, tissue sections from turkeys sacrificed at 1, 2, 3, 4, 5, 5, 7, 9, and 12 days post-inoculation (dpi) were processed as described above, deparaffinized with Citrisolv, antigenic sites exposed by microwaving the tissues for 5 minutes in a citrate buffer, then incubated with primary antibody diluted 1:500 in phosphate buffered saline containing 0.1% Tween-20 (PBST) overnight at 4°C. After incubation in primary antibody, the slides were washed in PBST, incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature (RT), washed in PBST, then incubated with a Alexa488-streptavidin-labeled antibody (Molecular Probes, Eugene OR) diluted 1:200 in PBST for 1 hour at RT. Slides were mounted in PBS+glycerol and fluorescence was examined on a motorized Zeiss Axioplan Ili equipped with a rear mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, and a Zeiss AxioCam B&W CCD camera. Fluorescence images were pseudocolored, and merged using OpenLabs 3.0 software (Improvision Inc., Lexington MA).

Co-Localization of TAstV-2 Antigen and Apoptosis. To determine if TAstV-2 induced cell death, intestinal sections from control or TAstV-2-infected turkey poulters were deparaffinized and antigenic sites exposed as described above, then incubated with terminal deoxynucleotide transferase labeled with tetramethylrhodamine red fluorescence (*In situ* End Labeling TUNEL analysis, Roche Molecular) for 1 hour at 37°C following manufacturer's instructions. Immediately following TUNEL staining, the sections were washed three times with PBST and stained for TAstV-2 as described above.

Statistics. Data comparing body weights and lymphoid organ weights were analyzed by one-way analysis of variance (ANOVA) and pairwise multiple comparison using the Student Newman-Keuls method (SigmaStat, Jandel Scientific, San Rafael, CA). Significance level was defined at $P < 0.05$.

Results

Propagation of TAstV-2 in Embryos.

Attempts to propagate TAstV-2 in cell culture using primary turkey embryo fibroblast, turkey embryo kidney cells, chicken embryo fibroblast, chicken embryo kidney cells, African Green Monkey kidney cells (Vero), mink lung epithelial cells (Mv1Lu), Madin-Darby canine and bovine kidney cells (MDCK and MDBK), a human colorectal adenocarcinoma cell line (Caco-2), and an ileocecal colorectal adenocarcinoma cell line (HCT-8), were unsuccessful. Therefore, specific pathogen-free (SPF) turkey embryos at 20 embryonic days of age were inoculated with a tissue filtrate prepared from healthy or TAstV-2-infected turkey poults and incubated for 1, 3 or 5 days at 39°C. Intestines were removed and tested for TAstV-2 RNA and replication by RT-PCR and *in situ* hybridization respectively. RT-PCR analysis on embryo intestines was positive for TAstV-2 at days 1 through 5 post inoculation. *In situ* hybridization showed extensive viral replication within 1 dpi. TAstV-2 replication increased until 3 dpi and then began to decrease by 5 dpi. No TAstV-2 *in situ* staining was detected in the control embryos. Interestingly at 5 dpi, TAstV-2-infected embryo intestines were enlarged, thin-walled, and distended. An immense accumulation of intestinal fluid was also observed in the intestines of TAstV-2-infected embryos but not the controls. These results demonstrate that turkey embryos support TAstV-2 replication and are a valuable source for *in vitro* propagation.

TAstV-2-Induced Disease.

Clinical Signs and Gross Lesions

Inoculation of naive poults with 10^6 genomic units of TAstV-2 resulted in 100% of the infected birds developing diarrhea within 24 hours of challenge that continued throughout the course of the 12 day experiment. Diarrhea was watery, yellow, frothy, mucus-filled, but did not contain undigested food or blood. Control animals had no diarrhea. In addition to the diarrhea, infected birds exhibited statistically significant growth depression as compared to uninfected controls ($p < 0.05$). At 5 dpi, there was a about 27% difference in the growth, and a 38% difference by 12 dpi. The TAstV-2-infected birds remained smaller throughout experiments extended to 28 dpi.

Upon necropsy, the intestines of infected poult were distended, dilated, and gasfilled. The intestines appeared to be three to five times the size of those of the noninfected controls. In addition to the macroscopic changes seen in the intestines, we noted that the bursa and thymus, and to a lesser extent the spleens, of the infected animals appeared reduced in size. To examine this further, these organs were removed, weighed, and compared to those of the mock-infected poult. Birds infected with TAsV-2 had a statistically significant decrease in the size of the thymus beginning 3 dpi and continuing through 9 dpi ($p < 0.05$). Calculating the differences as a ratio of organ weight to body weight we found, at 3 dpi, the thymus of the TAsV-2-infected group was 36% smaller than the control group and 52% smaller at 9 dpi. However, by 12 dpi, there was no difference in the relative thymic size suggesting these changes were transient. There were no statistically significant differences in the sizes of the bursa or spleen as compared to controls.

Histopathological Lesions

To investigate the histologic changes resulting from TAsV-2 infection, tissues were examined by routine hematoxylin and eosin staining and light microscopy. In spite of the severe diarrhea, the intestinal lesions were mild. By 2 dpi, there were scattered single degenerating villous epithelial cells, predominantly in the basal portions of the villi. These degenerating cells were present through 9 dpi. Crypt hyperplasia was very mild at 3 dpi and continued through 12 dpi. By 5 dpi there was a minimal amount of mononuclear inflammatory infiltrate in the lamina propria that resolved by 12 dpi. Because of the gross changes seen in the thymus we also examined extra-intestinal tissues; bursa, spleen, pancreas, thymus, liver, kidney, bone marrow, skeletal muscle, and blood. No remarkable histologic changes were noted in any of these tissues. No lesions were seen in any of the control tissues. These findings demonstrate that TAsV-2 infection resulted in severe diarrhea, growth suppression, and reduction in thymic mass in the absence of widespread inflammation or cellular damage.

Localization of TAsV-2

TAsV-2 was originally isolated from the thymus suggesting that TAsV-2 is present outside the intestines (Schultz-Cherry et al., 2000). The distribution of

TAstV-2 was examined at different times post-infection by RT-PCR, isolation of infectious virus, immunofluorescence, and *in situ* hybridization. Not surprisingly, infectious virus could be isolated from the feces and intestines at all time points in the experiment from day 2 onward; however, the levels of virus in the feces at 1 dpi were below the level of detection by RT-PCR. TAstV-2 RNA was also detected by RT-PCR in the thymus, bursa, spleen, liver, kidney, pancreas, skeletal muscle, bone marrow, and in the plasma fraction of infected birds, generally at 3 and 5 dpi; and the thymus and spleen were still positive at 7 dpi. Infectious virus could be isolated from all of the samples generally between 3 to 7 dpi. The presence of TAstV-2 outside the intestines was also detected by immunofluorescence. Mild, limited TAstV-2 capsid staining was detected in all tissues examined, most consistently between 3 and 5 dpi. No staining was observed in control tissues. Although there was infectious virus and viral antigen staining in extra-intestinal tissues, *in situ* hybridization data suggested that astrovirus replication was limited to the intestines. No replicating virus was detected in representative extra-intestinal tissues (thymus, bursa, and spleen). *In situ* staining of the TAstV-2 genome in the intestines was generally found in the deep edges of the villi and not in the crypts. A similar staining pattern for TAstV-2 capsid protein was observed, with antigen detected in the cytoplasmic portion of specific enterocytes at the mid-region of the villi.

20 TAstV-2 Infection Does Not Increase Cell Death

The lack of histologic lesions in the intestines of TAstV-2-infected animals was surprising given the levels of viral replication and diarrhea. To determine if TAstV-2-infected cells undergo cell death, intestinal sections from control and infected poult were double-labeled for TAstV-2 capsid protein and cell death using TUNEL analysis. Not surprisingly, there was a great deal of TUNEL staining in both control and TAstV-2-infected intestines. In contrast astrovirus staining was found only in the cytoplasm of enterocytes of infected but not control intestines. Double-labeling the tissues resulted in no overlap of TUNEL-positive cells with TAstV-2-infected cells, suggesting that astrovirus replication does not result in an increase in cell death. Identical results were observed in TAstV-2-infected embryos

(data not shown). These experiments suggest that TAstV-2 does not increase cell death, which supports the histopathology observations.

Discussion

In these studies, an *in ovo* method to propagate high titers of infectious virus and a small animal model that will be useful to further understand astrovirus pathogenesis and the host response to infection, were described. The present studies examined the pathogenesis of astrovirus infection including the kinetics of astrovirus replication, the location of the virus and its ability to localize to extra-intestinal sites, and, most surprisingly, the induction of diarrhea in the absence of either cellular damage or an increased inflammatory response.

All of the human astrovirus (HAstV) strains were adapted to replicate in cell lines (Briner et al., 2000; Lee et al., 1981; Taylor et al., 1987). In contrast, TAstV-2 did not propagate in cell lines that support HAstV replication, or in primary turkey or chicken cells. Fortunately, TAstV-2 can be propagated in turkey embryos. Inoculation of TAstV-2 in the yolk sac of 20-day-of-age turkey embryos resulted in productive viral replication, accompanied by an accumulation of fluid in the intestines of infected embryos. This fluid typically contains 10^{11} viral genomic units/ml as determined by CQ RT-PCR. Limiting dilutions in embryos followed by immunofluorescent staining for the viral capsid protein suggested that the fluid contained at least 10^9 infectious viral particles/ml.

TAstV-2 is highly infectious and extremely stable in the environment (Schultz-Cherry et al., 2001); therefore, control birds had to be housed in separate rooms to avoid cross contamination. Additionally, placing naïve poult in contact with infected birds or in cages that previously housed TAstV-2 infected birds resulted in immediate infection and diarrhea. Similar to mammalian astroviruses, younger animals are more susceptible to TAstV-2 infection. Infecting older naïve birds with TAstV-2 induced diarrhea; however, the duration of viral replication and the clinical signs were reduced in older animals. Infecting naïve poult with TAstV-2 resulted in diarrhea in 100% of the birds within 24 hour post-infection. Infected poult had a reduced growth rate, and remained significantly smaller than controls throughout the experiment. In addition to the growth depression, infected poult also

had significantly reduced thymus weights, although this difference had resolved by the end of the experiment. The mechanism for the reduced growth rate and undersized thymus is not understood; however, both are likely directly related to the diarrhea. Infected birds likely suffer some nutritional deficiencies. Infected birds consumed the same amount of feed as the age matched controls, but did not gain weight at the same rate. In additional studies, birds given nutritional additives did not have as severe weight loss or changes to the thymus.

TAstV-2 RNA and infectious virus were detected in every tissue examined, including the blood. To confirm that TAstV-2 RNA and infectious virus present in nonintestinal tissues was independent of contaminating blood, tissues were washed extensively in PBS or incubated overnight in large volumes of formalin followed by a second 48 hour incubation in PBS prior to processing. Thus, it is unlikely the TAstV-2 is due to contaminating blood. Additionally, we confirmed the presence of TAstV-2 in nonintestinal organs by immunofluorescent staining for the capsid protein. The distribution of viral antigen and RNA throughout non-intestinal organs peaked at 5 dpi then waned. By 12 dpi, only the intestine contained virus. There was limited capsid staining in lymphoid areas of the thymus and bursa and in the kidney epithelia. However, most of the TAstV-2 capsid staining in the extra-intestinal tissues was associated with vasculature. Previously it was unknown if astroviruses induced viremia. In this study, TAstV-2 RNA and low titers of virus were detected in plasma samples from infected poult. Many viruses induce viremia during which the viruses circulate in the blood, serum, or white blood cells (WBCs) and are spread to target organs to initiate infection (Mims et al., 1989). The mechanism by which TAstV-2 enters the blood stream and spreads to extra-intestinal organs is unknown. Studies with astrovirus in lambs and calves suggested a possible role for macrophages, Peyer's patches, and M cells in infected animals (Behling-Kela et al., 2002). However, macrophages isolated from the spleens of TAstV-2-infected poult did not contain infectious virus. Collectively, these results suggest that viremia occurs following TAstV-2 infection and that the TAstV-2-positive sera contain infectious virus.

Although, extra-intestinal tissues contained TAstV-2 antigen and RNA, only the intestine appeared to support viral replication as determined by *in situ* hybridization. Limited replication was observed in the cecal tonsils and distal small intestine within 1 dpi. By 3 dpi, replication was pronounced in the cells of the mid-
5 villus of the cecal tonsils and distal small intestine (duodenum) with expansion to the epithelium of the large intestine and small intestine. By 9 dpi, only minimal viral replication was observed (Behling-Klia et al., 2002).

Many enteric pathogens induce diarrhea by destroying enterocytes in the villous epithelium ultimately leading to cell death and villous atrophy (Lundgren et
10 al., 2000). This does not appear to be the case with TAstV-2. In spite of the diarrhea, there were only minimal to mild histologic changes in the intestines during TAstV-2 infection. The lack of substantial histologic changes noted in the intestines was supported by TUNEL analysis. TUNEL staining demonstrated that cell death was not increased during infection, either in general or specifically in TAstV-2
15 infected cells. Similar results were obtained using the apoptosis-specific antibody, caspase 3 (data not shown).

Example II

Methods

20 Recombinantly-expressed TAstV-2 capsid protein was generated utilizing the Bac-To-Bac Baculovirus Expression System (Invitrogen) following the manufacturer's instructions. Briefly, the TAstV-2 capsid gene was subcloned from pcDNA3.1-TAstVcap10 into the pFastBacTM HTa expression vectors (Invitrogen) to generate pFastBacHT/TAstV-2capsid. The resultant plasmid was screened by
25 sequence analysis to ensure generation of the fusion protein, and to confirm the integrity of the TAstV-2 gene. The construct was recombined into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome via DH10Bac cells. The recombinant baculovirus (rAcNPV/TAstV-2capsidHis) was propagated in serum-free media (SFM)-adapted Sf9 or Sf21 insect cells and used to express TAstV-2
30 capsid protein. The infected cells were harvested at different times post-infection and monitored for capsid protein expression by Western blot analysis and

immunofluorescence microscopy using TAstV-2-specific antibodies. Specifically, baculovirus infected insect cells were lysed with 50 mM Tris (pH 8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% NP40. Cells were frozen and thawed twice and cell debris removed by centrifugation. His-tagged rTAstV-2

5 capsid protein was purified using Ni-NTA agarose beads (Qiagen) following the manufacturer's instructions. Affinity purified His-tagged protein was purified over a D-Salt Excellulose GF-5 Desalting Column (Pierce) to remove the imidazole elution buffer, and samples were checked for protein by SDS PAGE and western blot using Penta-His Ab (Qiagen) or anti-KHL IgG sera (see below).

10 TAstV-2 capsid peptides

Three peptides derived from predicted amino acid antigenicity and surface probability analysis were synthesized commercially (Invitrogen) corresponding to amino acid positions 32-47 (RSRTKKTVKIIEKKPE, RSR; SEQ ID NO:3), 194-221 (HPRSALGPRQGWWNVDPGD, HPR; SEQ ID NO:4) and 676-691

15 (KHLEEEKNYWKNQCER, KHL; SEQ ID NO:5). These peptides were used to stimulate HD11 cell, in soluble form, immobilized on microtiter plates, or cross-linked using disuccinimidyl suberate (DSS, Pierce). HD11 cells (1×10^5 /well) were stimulated with 1-25 μ g of peptides or bovine serum albumin (BSA) in each of the above forms.

20 Results

Recombinant TAstV-2 capsid protein (rTAstV-2cap) was produced in the baculovirus expression system. Western blot analysis and electron microscopy confirmed that recombinant capsid protein was expressed in infected insect cells (data not shown). In particular, high levels of astrovirus capsid protein were
25 expressed at 48 hours to 72 hours post-infection (hpi).

His-tagged TAstV-2 capsid protein was affinity purified and added to HD11 cells. The addition of 1 μ g of affinity purified rTAstV-2cap to HD11s stimulated NO production. NO levels were similar in cells treated with purified TAstV-2.

These data demonstrated that the TAstV-2 capsid protein was sufficient to stimulate
30 expression of NO.

Cells were also treated with peptides derived from the TAstV-2 capsid sequence. These peptides were selected based on surface probability and antigenicity index analysis, as well as sequence conservation. HD11 cells were treated with the peptides in soluble, bound, and cross-linked forms. None of the peptides stimulated NO production regardless of form. In addition, pre-incubating TAstV-2 with purified IgG specific to these peptide sequences failed to inhibit NO activity when added to HD11 cells, and pre-incubating the cells with the peptides did not inhibit binding. These results suggested that these peptides did not represent the cellular binding regions of the capsid protein.

Example III

Methods

Cell Culture. Serum free adapted Sf9 (*Spodoptera frugiperda*) cells (Invitrogen) were grown in Sf-900 II SFM (Invitrogen), in 25 ml suspension cultures, using plastic 200 ml Erlenmeyer flasks, on an orbital shaker at 145 rpms, at 27°C. Cells were grown to a density of 2×10^6 cells/ml and 98% viability, and passaged at a cell density of 5×10^5 cells/ml.

Recombinant baculovirus. Recombinant baculovirus expressing the TAstV-2 capsid protein was generated utilizing the Bac-To-Bac Baculovirus Expression System (Invitrogen) following the manufacturer's instructions (see Example II). An additional construct was created from pFastBacHT/TAstV-2capsid, in which the histidine tag was removed. Briefly, pFastBacHT/TAstV-2capsid was digested with *Nco* I (Invitrogen), *Rsr* II (Invitrogen), and Mung Bean S1 nuclease (New England Biolabs), and re-ligated using T4 ligase (Invitrogen). The resultant plasmid was screened by sequence analysis. The constructs were each recombined into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome via DH10Bac cells. The recombinant baculoviruses (rAcNPV/TAstV-2capsidHis, and rAcNPV/TAstV-2capsid) were propagated in serum-free media (SFM)-adapted Sf9 insect cells.

Antigen. Sf9 cells were seeded in 96 well plates at a concentration of 1×10^5 cells/well, and cultured at 27°C for 2 hours. After cells attached to the wells,

the culture media was removed and cells were infected with recombinant baculovirus or wild-type AcNPV (Paul Friesen, University of Wisconsin, Madison) at a multiplicity of infection (MOI) of 5. All wells were brought to a final volume of 100 µl in Sf-900 II SFM, and cells were incubated for 72 hours at 27°C.

- 5 Following incubation, the culture media was removed and cells were washed with 200 µl of PBS. Cells were then fixed in cold methanol:acetone (1:1) for 10 minutes. Fixative was removed, the cells were washed with PBS, then PBS was removed, and the plates stored at -20°C until needed.

- Anti-TAstV-2 Immunofluorescence Assay. Fixed, frozen infected insect
- 10 cells were warmed to room temperature, and rinsed once with PBS. Test serum from unknown turkeys was diluted 1:10 in PBS, and 25 µl added to both a rAcNPV/TAstV-2capsid infected well and an AcNPV infected well (negative control). As an assay control, a rabbit polyclonal sera generated against a peptide derived from the TAstV-2 predicted capsid sequence (amino acids 676-691, an
- 15 “anti-KHL IgG” sera), was diluted 1:750 in PBS and added to both a positive and negative control well, respectively. The plate was incubated for 1 hour at room temperature. Following incubation, the wells were washed with 200 µl of PBS three times. Following the final wash, PBS was removed, and secondary antibody added. Unknown turkey samples were detected using secondary Goat Anti-Turkey
- 20 IgG(H+L)-FITC (Southern Biotech) at a dilution of 1:100 in PBS. The assay control wells, were detected using an anti-rabbit IgG-rhodamine (Jackson Labs) secondary antibody diluted 1:750. Antibodies were added in 25 µl total volume and incubated at room temperature for 1 hour. Following secondary antibody binding, wells were washed with 200 µl of PBS three times, and once with water. Water was
- 25 removed and 10 µl of PBS: glycerol (1:1) added to each well. Cells were then examined for fluorescence using an inverted UV microscope. The presence of TAstV-2 capsid protein specific antibodies in the unknown turkey serum samples was determined by comparing the level of FITC fluorescence between test well (rAcNPV/TAstV-2capsid infected Sf9 cells) and negative control well (wild-type
- 30 AcNPV infected Sf9 cells).

Samples. Feces and intestines were collected from turkey poult infected with turkey astrovirus-2 or PEMS inoculum at 5 days post-infection (dpi). At 2 weeks post-infection, serum was collected and used for the serologic assay. In addition, intestines from 3 to 5 birds/ PEMS-positive flock at 1 week of age and serum from
5 the same flocks 3 weeks later, were collected.

Results

To determine whether recombinant cells expressing turkey astrovirus-2 capsid protein could detect anti-turkey astrovirus-2 antibodies present in a physiological sample, serum was collected from turkey poult infected with turkey
10 astrovirus-2 or PEMS inoculum, and from a PEMS-positive flock. In addition, cRNA was isolated from the feces or intestines of PEMS-infected turkey poult and tested for the presence of turkey astrovirus-2 using a RT-PCR test (Example I).

Some of the results are shown in Table 3. Thus, the serological assay accurately detects the presence of turkey astrovirus-2 infected birds.

Table 3. Comparison of Turkey Astrovirus-2 Serologic Test to RT-PCR

Samples	Total # of samples tested ^a	# Positive by Serologic Test ^b	# Positive by RT-PCR ^c
Experimental Study 1	5	3	4
Experimental Study 2	8	6	6
Experimental Study 3	5	5	5
Samples from Commercial Turkey Flocks ^d	6	4	6

- 10 ^aFeces and intestines were collected from turkey poultts infected with TAstV-2 or PEMS inoculum at 5 days post-infection (dpi). At 2 weeks post-infection, serum was collected and used for the serologic assay.
- ^bSerum was tested for the presence of astrovirus antibodies using insect cells infected with baculovirus expressing TAstV-2 capsid protein.
- 15 ^cRNA was isolated from the feces or intestines of PEMS-infected turkey poultts and tested for TAstV-2 using described RT-PCR tests.
- ^dA turkey company in North Carolina with PEMS-positive flocks collected intestines from 3 to 5 birds/flock at 1 week of age and serum from the same flocks 3 weeks later.

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30 All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification, this invention has been described in

relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the
5 invention.